Povidone–iodine induced cell death in cultured human epithelial HeLa cells and rat oral mucosal tissue

So Sato, MD

This is an Accepted Manuscript of an article published by Taylor & Francis in Drugs and Chemical Toxicology on 2014, available online: http://www.tandfonline.com10.3109/01480545.2013.846364.

Department of Otolaryngology, Fukushima Medical University, 1 Hikarigaoka, Fukushima City, Fukushima Pref, Japan

Department of Otolaryngology, 1 Hikarigaoka, Fukushima City, Fukushima Pref, 960-1295 Japan

Tel.: +81-24-547-1325

Fax: +81-24-547-1325

E-mail: miklikov@hotmail.com

Key terms: apoptosis, necrosis, toxicity, fluorescent staining, HeLa cells, rat oral mucosa

Abstract

Context

Although Povidone–iodine (PVP-I) has been used as a gargle since 1956, its effectiveness and material safety have been remained controversial.

Objective

The aim of this study was to investigate the toxicity of PVP-I to epithelial cells in a concentration range significantly lower than that used clinically.

Material and Methods

Study design was in vitro laboratory investigations and in vivo histological and immunologic analysis. We examined the effects of PVP-I at concentrations of 1×10^{-2} to 1×10^{3} μM and 1×10^{-4} to 1×10 μM on HeLa cells,

as a model of epithelial cells, and rat oral mucosa, respectively, after one or two days of exposure. Annexin V-FLUOS was used to distinguish live, apoptotic, and necrotic cells. TUNEL method was also used to observe whether apoptotic epithelial cells exist in rat oral mucosa after one day of exposure of PVP-I.

Results

HeLa cells developed concentration-dependent cytotoxicity, and epithelium of the rat oral mucosa were thinned in a concentration-dependent manner. HeLa cell apoptosis increased after 1×10^{0} μM PVP-I exposure for two days. In the TUNEL method, many apoptotic epithelial cells were observed in the rat oral mucosa following one day of exposure to diluted 1×10^{-2} μM PVP-I, but minimal apoptotic epithelial cells were observed using 1×10^{-3} μM PVP-I.

Discussion and Conclusions

Our findings suggest that exposure to PVP-I, of which concentrations are even lower than those used clinically, causes toxicity in epithelial cells. This knowledge would help us better understand the risk of the use of PVP-I against mucosa.

Introduction

PVP-I is a complex of polyvinylpyrrolidone and iodine developed by Shelanski in 1956 as an oral cavity gargle and disinfectant. The anti-bacterial mechanism of the complex of polyvinylpyrrolidone and iodine (Povidone–iodine, PVP-I) involves the break down of the bacterial cell membrane proteins by H_2OI^+, which is generated from available I_2 [1]. PVP-I also has sterilizing properties against viruses and eumycetes, and some reports indicate its in vitro efficacy against severe acute respiratory syndrome (SARS) [2] and the H5N1 influenza virus [3].

The application of PVP-I to a topical wound or the use of iodine radiocontrast agents can induce nephropathy and iododerma. Iododerma is a type of dermatitis caused by iodine toxicity or allergy that leads to intraepidermal separation with necrosis and pseudoepitheliomatous hyperplasia as a local or systemic disease [4, 5, 6]. Nephropathy and iododerma caused by PVP-I exposure is due to cell necrosis [4, 5]. PVP-I toxicity in fibroblasts and keratinocytes has been reported [7–10], although these studies failed to quantify the toxicity. There has been one report on

the damage of corneal epithelial cells by PVP-I, however, the concentration of PVP-I was relatively high [11]. It reported that hearing loss was observed in infant, and adult guinea pig when 10% PVP-I was inserted into their middle ear for 24 hours [12]. Further studies into the toxic effects of PVP-I using epithelial cells are necessary to re-evaluate its clinical usage. For this purpose, we used rat oral mucosa and HeLa cells, which are widely used as a model of epithelial cell for investigation of cellular damage mechanism.

Conventionally, PVP-I has been used to reduce the bacterial count for disinfection. Generally, exposure of the mucosa to a high concentration of PVP-I causes necrosis, whereas exposure to a low concentration induces apoptosis. Thus, it is necessary to determine the amount of time required to cause such damage. The aim of this study is to evaluate the dose– and –time–dependence of PVP-I toxicity against HeLa cells and the epithelium, and to determine whether concentrations showing toxicity are, significant lower than those used clinically. With such data, we would be able to identify the degree of PVP-I toxicity when clinical side effects of PVP-I occur.

Materials and Methods

Chemicals

A solution of PVP-I (10%) containing100 mg PVP-I in 1 ml, with a 10% content of available iodine was obtained from Mylan (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) and 0.5% trypsin solution were obtained from Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Life Technologies (Tokyo, Japan), and 10% FBS (v/v) was supplemented to DMEM immediately prior to use. Phosphate-buffered saline (PBS: 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH7.4) and two fluorescent dyes, propidium iodide (PI, 1 μg/ml, used to stain dead cells) and 4',6-diamidino-2-phenylindole (DAPI, 1 μg/ml, used to stain nucleus of sections) were also obtained from Wako. Annexin V-FLUOS solution, which can detect whether cells have phosphatidylserine on the cell surface, an event found in apoptosis, and is used to stain apoptotic and necrotic cells, was obtained from Roche (Mannheim, Germany). Calcein-AM, a dye used to stain live cells was obtained from Dojindo Molecular Technologies, Inc., (Rockville, MD, USA).

-

Cells culture

HeLa cells were acquired from JCRB cell bank (JCRB 9004). The seeding densities was 67000/ml, 28000/ml and 16000/ml, and the seeding volume per well was 100 µl. One day after seeding, the wells were exposed to PVP-I (1×10^{-2} to 1×10^{3} µM), which had been diluted with DMEM containing 10% FBS. The plates were further cultured for one or two days at 37°C in a 5% CO_2 incubator (BNA-111, Tabei Espec Corp, Osaka, Japan). The cell incubation time of one or two days was determined based on data regarding the onset time of iododerma and nephropathy pathogenesis [4, 5]. Individual experiments were performed independently (as singles) in wells with the same concentration.

Tissue culture

Fifteen male nine-week-old Sprague-Dawley rats (SD rats, Charles River Laboratories Japan, Yokohama, Japan) were used in this study. All rats were sacrificed and four oral mucosa samples (5×5 mm) were obtained from each rat. This study was carried out in strict accordance with the

recommendations of the Animal Research Committee of Fukushima Medical University. The protocol was approved by Fukushima Medical University, Fukushima, Japan (Permit Number 22025). To anesthetize the rats, a mixture of medetomidine (0.15mg/kg BW), midazolam (0.20mg/kg BW), and butorphanol tartrate (0.25mg/kg BW) was injected intravenously. The rats were then sacrificed by intravenous injection of a mixture of medetomidine (0.45mg/kg BW), midazolam (0.60mg/kg BW), and butorphanol tartrate (0.75mg/kg BW). PVP-I (1×10^{-4} to 1×10^{1}μM) were diluted with DMEM containing 10% FBS and the mucosa samples were exposed to the PVP-I in the culture dishes. The mucosa samples were then cultured further for one or two days at 37°C in a CO_2 incubator at 5 % CO_2. The same experiments were repeated twice to validate the results.

Miroscopes and cameras

An inverted microscope (IX71, Olympus, Tokyo, Japan) and a cooled CCD camera (pixera penguin 150CLM, Pixera Corporation, CA, USA) with capture software (Pixera InStudio, Pixera Corporation, CA, USA) were used for HeLa cell experiments.

An upright microscope (BX50, Olympus, Tokyo, Japan) and a single chip color CCD camera (DP70, Olympus, Tokyo, Japan) with capture software (DP controller, Olympus, Tokyo, Japan) were used for tissue sections.

Determination of cell density (HeLa cells)

After the HeLa cells were exposed to PVP-I solutions for one or two days, PVP-I solutions were removed, and the cells were washed with PBS. The wells were then filled with 0.5% trypsin to detach the cells. The number of cells in each well was counted using a Fuchs-Rosenthal Counting Chamber (KA103, Minato Medical, Tokyo, Japan), and analyzed.

Detection of apoptotic/necrotic cells (HeLa cells)

The seeding density used for the detection of apoptotic and necrotic cells in HeLa cells was 28000/ml. After the HeLa cells were exposed to PVP-I for one or two days, the solutions were removed, and the cells were washed with PBS, then incubated with PI and Annexin V-FLUOS in fresh PBS for 30 min to stain. Images of every well were taken (six images for every

concentration, 60 images for each day, 120 images in total). Every captured image contained more than 100 cells, and the fluorescence threshold of stain/non-stain was determined with ScionImage (Scion corp., MD, USA). All cells in view were identified as live (unstained), apoptotic (green), and dead (green and red), and each proportion was calculated, and analyzed.

Confirmation of live cells with calcein-AM (HeLa cells)

The seeding density used in the calcein-AM assay of the HeLa cells was 28000/ml. To confirm whether stained cells were actually live cells, calcein-AM was used to complement the determination of live cells. After HeLa cell exposure to PVP-I for two days, the PVP-I solutions were removed, and cells were washed with PBS, then incubated with PI and calcein-AM for 15 min. The images were taken under a phase contrast microscope.

Comparison of epithelial thickness (rat oral mucosa)

After rat oral mucosa exposure to PVP-I, the mucosa samples were washed with PBS. Formalin-fixed paraffin embedded (FFPE) sections

(thickness 5 μm) were then prepared and stained with haematoxylin and eosin. Pictures of the sections were acquired by a microscope with a camera. The epithelial thickness was measured from the epidermal basement membrane to the stratum superficiale using 20 vertical lines, which were observed in a microscopic field, and the mean was calculated at every concentration, using Adobe Photoshop CS5 (Adobe Systems Incorporated, CA, USA). Four slides were measured and analyzed at every concentration.

Detection of apoptotic cells in rat oral mucosa by the TUNEL method

To identify the apoptotic cells, the sections were stained using the TUNEL method with the In Situ Cell Death Detection Kit-TMR red (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. In brief, we selected one arbitrary section from rat oral mucosa for each PVP-I concentration after one day of exposure. The sections were transferred through xylene, graded ethanol, and water, before being irradiated by microwave at 350 W for 5 min. The DNA end was labeled with10 μl of TUNEL reaction solution. All cells were counterstained with

DAPI. The images were recorded by the inverted microscope's camera equipped with filters.

Statistics

All data was analyzed using non-repeated ANOVA/Dunnett post hoc test.

Results

HeLa cells

HeLa cells grew steadily in a PVP-I free environment; however, growth was inhibited to a significant degree by one-day exposure to concentrations of 3×10^{-2} μM of PVP-I or greater (Figure 1a–c) and, by two-day exposure to concentrations of 1×10^{-2}μM or greater (Figure 1a, 1b) or 3×10^{-2} μM or greater (Figure 1c). The same results were found for every seeding. In the figure, each column represents the mean value with SD. and each concentration was statistically compared to the control for each respective day. We confirmed these results for every seeding density (Figure 1).

Figures 2a and 2b show the proportion of live, apoptotic, and necrotic cells at the specified concentrations for Days 1 and 2, respectively. As shown in Figure 2a, the proportion of live cells decreased at the concentrations of 1×10^{1} μM or greater, apoptotic cells increased at the concentrations of 1×10^{2} μM or greater, and necrotic cells increased at a concentration of 1×10^{3} μM after one day of exposure. On the other hand, the proportion of live cells decreased at the concentrations of 1×10^{0} μM or greater, apoptotic cells increased at the concentrations of 1×10^{0} μM or greater, and necrotic cells increased at the concentrations of 1×10^{2} μM or greater after two days of exposure, as shown in Figure 2b. The proportion of apoptotic cells initially increased with the increase in the PVP-I concentration, but eventually reached a point where further increases in the PVP-I concentration caused this proportion to decrease (Figures 2a and 2b). Figures 2c, 2d, and 2e show that live cells were not stained by Annexin V-FLUOS, whereas, both apoptotic and necrotic cells were stained (green). Necrotic cells were also stained by PI (red).

PVP-I was observed to cause dose-dependent cytotoxicity against the HeLa cells after exposure for two days (Figure 3a–f). The HeLa control cells

kept normal cytoplasm, while those exposed to 1×10^{1} μM PVP-I had a blister-like cytoplasm (Figure 3g–i). We also saw similar results for every seeding density. Figure 4 is the summary of the effect transition of HeLa cells exposed to PVP-I for one or two days..

Rat oral mucosa

On incubating the control and mucosa with PVP-I (1×10^{1} μM), a similar epithelial structure was observed on Day 1 (Figure 5a–c). However, on Day 2, the epithelium incubated with PVP-I (1×10^{1} μM) was much thinner than that of the control. The entire superficial and medium layers were absent, and only the stratum basale remained. The epithelium was not only thinner but also morphologically damaged (Figure 5d–e). PVP-I functioned in a time-and concentration-dependent manner (Figure 5f). The epithelium was statistically thinner after one day of exposure to PVP-I to concentrations of 1×10^{-1} μM or greater for one day, and 1×10^{-2} μM or greater for two days of exposure.

Following the staining of rat oral mucosa samples by using the TUNEL method, Minimal apoptosis was observed in the control and PVP-I

(1×10^{-3} μM) model after one day of exposure, however many apoptotic cells were observed in the PVP-I (1×10^{-2} μM) model as shown in Figures 6. We also observed minimal apoptosis in the Day 1 PVP-I (1×10^{-4} μM) model, but many apoptotic cells were detected in the PVP-I (1×10^{-1}, 1×10^{0}, and 1×10^{1} μM) models.

Discussion

The present study shows that PVP-I induces HeLa cell apoptosis and necrosis in a dose-dependent manner. The clinical purpose of gargling with PVP-I is sterilization, but in our study, the purpose was to reveal the toxicity of PVP-I to cells and tissues. We used three cell seeding densities in the evaluation of the number of cells. The reason for this is because cell density affects the distribution of cell cycle during incubation, and cell sensitivity to cytotoxic agents depends on cell condition such as cell cycle. The results of the three cell seeding density were almost the same, thus we decided to use single density (28000/ml) from that point on. In the preliminary experiment, we found the cytotoxicity of HeLa cells and epithelial thinning of rat oral mucosa at a lower PVP-I concentration than that used clinically. We therefore defined a PVP-I concentration range from very lower than the clinical use to that close to the clinical use. This

concentration range was chosen in order to observe adverse effects on the mucosa by long-period of exposure to lower concentration of PVP-I. We also found that rat oral mucosa had epithelial damage in lower PVP-I concentrations range, in contrast HeLa cells were damaged in the higher concentrations range. The discrepancy between the PVP-I concentrations used in the HeLa cells and rat oral mucosa may be explained by the differences in malignancy. HeLa cells are malignant cells; therefore, possibly more resistant to PVP-I than normal cells (rat oral mucosa).

Our results are concordant with previous reports, which show that once epithelial cells are stimulated by a cytotoxic agent, their number immediately decreases upon reaching threshold of toxicity [13]. After considering all the background information on PVP-I for this study, we hypothesized that its greatest merit was sterilization and its most significant contraindication was epithelial injury. In previous studies, apoptotic and necrotic HeLa blebs elicited by H_2O_2 were compared in terms of dynamics. Apoptotic blebs appeared in a few minutes and required millimolar peroxide concentrations. Necrotic blebs, however, appeared much later, prior to cell permeabilization, and also required millimolar peroxide concentrations [14]. In our study, the rat oral mucosa was also found to be

damaged by exposure to PVP-I, showing significant thinning of the epithelium, which was apparent after one or two days of exposure.

PVP-I is used clinically as a skin disinfectant at a concentration of 4×10^4 μM, whereas PVP-I gargle concentrations range from 1.9×10^3 to 3.7×10^3 μM, both of which are much higher than the 1×10^{-2} μM concentration that induced apoptosis in the rat oral mucosa of the present study. Although this appears to suggest that PVP-I oral disinfectant usage will undoubtedly lead to epithelial damage, gargling for up to 10 s exposes the epithelium for a much shorter period of time than the one or two days of exposure of mucosal mucosa samples in the present study. Therefore, the effects of PVP-I may be both time- and concentration-dependent.

Previous work has suggested that, among healthy people, simply gargling with water is more effective in preventing upper respiratory infections than using PVP-I [15], which may even be toxic to healthy individuals. Furthermore, a short period of gargling with concentrated PVP-I may be enough to induce a cytotoxic effect. PVP-I cytotoxicity was also observed in other studies on several different primary cultured cells undergoing necrosis following PVP-I exposure [7, 16]. Rabbit corneas

became edematous three days after 10 drops of 1.5–2% (6×10^3 to 8×10^3 μM) PVP-I were inserted into their eyes [17].

The efficacy of PVP-I in mucosal therapy is controversial. One study, for example, showed that PVP-I was as effective as topical ciprofloxacin for chronic suppressive otitis media [18], where the PVP-I group had lower mucositis scores than the salt/soda and chlorhexidine group [19]. Furthermore, 68% of non-specific vaginitis caused by *Gardnerella vaginalis* was improved with a PVP-I pessary [20]. On the other hand, 5% PVP-I was less effective than 5% natamycin in the treatment of fungal keratitis caused by *Fusarium solani* in a rabbit model [21].

One report suggested that no PVP-I cytotoxicity was observed on intact skin [22], and the another speculated the safety and effectiveness of PVP-I for the treatment of wounds [23]. The application of PVP-I to topical wounds or the use of iodine radiocontrast agents induce nephropathy and iododerma [4, 5, 6, 24–26], the latter being defined as a local and systemic eruption of follicular papules and pustules or a granulomatous lesion caused by iodine toxicity or sensitivity. If the cause of iododerma is PVP-I toxicity, then systemic eruptions may result from hematogenous locomotion of PVP-I.

We complied these findings as a basic set of data to obtain balanced use of PVP-I.

Conclusion

The growth of HeLa cells was suppressed after one or two days exposure to PVP-I concentrations lower than those used clinically. With those PVP-I concentrations, apoptosis and necrosis were induced in the HeLa cells and rat oral mucosa after one or two days of exposure. Epithelial thinning occurred when epithelial cells and tissues were exposed to PVP-I continuously for one or two days in this study. Our study helps us understand the risk of PVP-I application to the mucosa even with lower concentrations than those used clinically.

Acknowledgments

This study was supported by a Grant-in-Aid for Young Scientists (B) (21791631) from the Ministry of Education, Culture, Sports, Science and

Technology, Japan. We would like to thank Professors Akihiro Hazama and Koichi Omori, and Dr. Masao Miyake for useful discussions and experimental support. We thank Etsuko Sato for her technical assistance.

Declaration of Interest section

The authors report no declarations of interest.

References

[1] Hsu YC, Nomura S, Krusé CW. (1965). Some bactericidal and virucidal properties of iodine not affecting infectious RNA and DNA. Am J Epidemiol, 82, 317-328.

[2] Kariwa H, Fujii N, Takashima I. (2006). Inactivation of SARS coronavirus by means of Povidone–iodine, physical conditions and chemical reagents. Dermatology, 212 Suppl 1, 119-123.

[3] Ito H, Ito T, Hikida M, Yashiro J, Otsuka A. (2006). Outbreak of highly pathogenic avian influenza in Japan and anti-influenza virus activity of Povidone–iodine products. Dermatology, 212 Suppl 1, 115-118.

[4] Manfro RC, Comerlato L, Berdichevski RH, Ribeiro AR, Denicol NT, Berger M, Saitovitch D, Koff WD, Goncalves LF. (2002). Nephrotoxic acute renal failure in a renal transplant patient with recurrent lymphocele treated with Povidone–iodine irrigation. Am J Kidney Dis, 40(3), 655-657.

[5] Massé M, Falanga V, Zhou LH. (2008). Use of topical Povidone–iodine resulting in an iododerma-like eruption. J Dermatol, 35, 744-747.

[6] Chang MW, Miner JE, Moiin A, Hashimoto K. (1997). Iododerma after computed tomographic scan with intravenous radiopaque contrast media. J Am Acad Dermatol, 36, 1014-1016.

[7] Nomura Y, Bhawal UK, Nishikiori R, Sawajiri M, Maeda T, Okazaki M. (2010). Effects of high-dose major components in oral disinfectants on the cell cycle and apoptosis in primary human gingival fibroblasts in vitro. Dent Mater J, 29, 75-83.

[8] Teepe RG, Koebrugge EJ, Löwik CW, Petit PL, Bosboom RW, Twiss IM, Boxma H, Vermeer BJ, Ponec M. (1993). Cytotoxic effects of topical antimicrobial and antiseptic agents on human keratinocytes in vitro. J Trauma, 35, 8-19.

[9] Smoot EC 3rd, Kucan JO, Roth A, Mody N, Debs N. (1991). In vitro toxicity testing for antibacterials against human keratinocytes. Plast Reconstr Surg, 87, 917-924.

[10] Balin AK, Pratt L. (2002). Dilute Povidone–iodine solutions inhibit human skin fibroblast growth. Dermatol Surg, 28, 210-214.

[11] Chou SF, Lin CH, Chang SW. (2011). Povidone–iodine application induces corneal cell death through fixation. Br J Ophthalmol, 95(2), 277-283.

[12] Ichibangase T, Yamato T, Miyagi M, Nakagawa T, Morizono T. (2011). Ototoxicity of Povidone-Iodine applied to the middle ear cavity of guinea pig. Int J Pediatr Otorhinolaryngol, 75(9), 1078-81.

[13] Obata S, Yamaguchi Y, Miyamoto T. (1990). Effects of etoposide (VP-16) on the survival and progression of cultured HeLa S3 cells through the cell cycle. Nippon Gan Chiryo Gakkai Shi, 25, 2484-2491.

[14] Barros LF, Kanaseki T, Sabirov R, Morishima S, Castro J, Bittner CX. (2003). Apoptotic and necrotic blebs in epithelial cells display similar neck diameters but different kinase dependency. Cell Death Differ, 10, 687-697.

[15] Satomura K, Kitamura T, Kawamura T, Shimbo T, Watanabe M, Kamei M, Takano Y, Tamakoshi A. (2005). Prevention of upper respiratory tract infections by gargling: a randomized trial. Am J Prev Med, 29, 302-307.

[16] Opitz I, Sigrist B, Hillinger S, Lardinois D, Stahel R, Weder W, Hopkins-Donaldson S. (2007). Taurolidine and Povidone–iodine induce

different types of cell death in malignant pleural mesothelioma. Lung Cancer, 56, 327-336.

[17] Jiang J, Wu M, Shen T. (2009). The toxic effect of different concentrations of Povidone–iodine on the rabbit's cornea. Cutan Ocul Toxicol 2009, 28, 119-124.

[18] Jaya C, Job A, Mathai E, Antonisamy B. (2003). Evaluation of topical Povidone–iodine in chronic suppurative otitis media. Arch Otolaryngol Head Neck Surg, 129, 1098-1100.

[19] Madan PD, Sequeira PS, Shenoy K. (2008). The effect of three mouthwashes on radiation-induced oral mucositis in patients with head and neck malignancies: a randomized control trial. J Cancer Res Ther, 4 (1), 3-8.

[20] Dattani IM, Gerken A, Evans BA. (1982). Aetiology and management of non-specific vaginitis. Br J Vener Dis, 58, 32-35.

[21] Oliveira LA, Takata TT, Shiguematsu AI, Melo Júnior LA, Gompertz OF, Sousa LB, Mannis MJ. (2008). Effect of topical 0.5% Povidone–iodine compared to 5% natamycin in fungal keratitis caused by Fusarium solani in a rabbit model: a pilot study. Arq Bras Oftalmol, 71 (6), 860-864.

[22] Niedner R. (1997). Cytotoxicity and sensitization of Povidone–iodine and other frequently used anti-infective agents. Dermatology, 195 Suppl 2, 89-92.

[23] Burks RI. (1998). Povidone–iodine solution in wound treatment. Phys Ther, 78, 212-218.

[24] Miranda-Romero A, Sánchez-Sambucety P, Esquivias Gómez JI, Martínez Fernández M, Bajo del Pozo C, Aragoneses Fraile H, Garcia-Munos M. (1999). Vegetating iododerma with fatal outcome. Dermatology, 198, 295-297.

[25] Sparrow GP. (1979). Iododerma due to radiographic contrast medium. J R Soc Med, 72, 60-61.

[26] Yavascan O, Kara OD, Sozen G, Aksu N. (2005). Allergic dermatitis caused by Povidone–iodine: an uncommon complication of chronic peritoneal dialysis treatment. Adv Perit Dial, 21, 131-133.

Figure Legends

Figure 1

Total number of HeLa cells after exposure to PVP-I. The growth of HeLa cells was suppressed to a statistically significant level after exposure at the PVP-I concentrations of 3×10^{-2} μM or greater for one day, and 1×10^{-2} μM or greater for two days. a) 67,000 cells/ml, b) 28000 cells/ml, c) 16000 cells/ml. * indicates significant difference vs. control for each day (non-repeated ANOVA/Dunnett post hoc test). Because cell density affects the distribution of cell cycle during incubation, and cell sensitivity to cytotoxic agents depends on cell condition such as cell cycle, we used three different cell seeding densities.

Figure 2

Proportion of live, apoptotic, and necrotic HeLa cells after exposure to PVP-I for one or two day (n = 6). Each column represents the mean value with SD. a) One day. Live cells decreased at the concentrations of 1×10^{1} μM or greater, apoptotic cells increased at the concentrations of 1×10^{2} μM or

greater, and necrotic cells increased to a concentration of 1×10^{3} µM. b) Two days. Live cells decreased at the concentrations of 1×10^{0} µM or greater, apoptotic cells increased at the concentration of 1×10^{0} µM, and necrotic cells increased at the concentrations of 1×10^{2} µM. All changes were statistically significant. Astarisks (*) indicate significant difference from the control (non-repeated ANOVA/Dunnett post hoc test). c) Live cells (arrowhead) were not stained, d) apoptotic cells (double arrows) were stained by Annexin V-FLUOS (green), and e) necrotic cells (arrow) were stained by both Annexin V-FLUOS (green) and PI (red).

Figure 3

HeLa cells after exposure to PVP-I for two days. a)-f) Many live cells were visible in the control (calcein-AM staining, green) while the 1×10^{3} µM PVP-I experiment showed only dead HeLa cells (PI staining, red). PVP-I was observed to cause dose-dependent cytotoxicity against HeLa cells after exposure for two days. g)-i) HeLa cells in the control and 1×10^{0} µM model had a normal cytoplasm, while those in the 1×10^{1} µM model had a blister-like cytoplasm.

Figure 4

The borders between no damage/growth inhibition, growth inhibition/apoptosis, and apoptosis/necrosis observed after HeLa cells were exposed to PVP-I. a) One day, the borders between no damage/growth inhibition, growth inhibition/apoptosis, and apoptosis/necrosis were approximately 3×10^{-2} μM, 1×10^{2} μM, and 1×10^{3} μM, respectively. b) Two days, those were approximately 1×10^{-2} μM, 1×10^{0} μM, and 1×10^{2} μM, respectively.

Figure 5

Epithelium after PVP-I exposure. Arrows indicate epithelial thickness. a) Day0 control. b), c) epithelium incubated with 1×10^{1} uM for one day was thinner than that of the control. d), e) epithelium incubated with 1×10^{1} uM for two days was thinner than that of the control. f) PVP-I appears to function in both time- and concentration-dependent manner. Epithelium was statistically thinner after exposure to PVP-I to concentrations of 1×10^{-1} μM or greater for one day, and 1×10^{-2} μM or greater for two days. Each

column represents the mean value with SD. The symbols indicate significant differences from the control. * for day 1 and # for Day 2 (non-repeated ANOVA/Dunnett post hoc test).

Figure 6

TMR red staining of rat oral mucosa after one day of PVP-I exposure with the TUNEL method. All cells were stained with DAPI (blue), and apoptotic cells were stained with TMR red (red). a) Control model: a few cells stained with TMR red were observed. b) 1×10^{-3} μM model: a few cells stained with TMR red were also observed. c) 1×10^{-2} μM model: numerous cells stained with TMR red were observed.

Figure 1

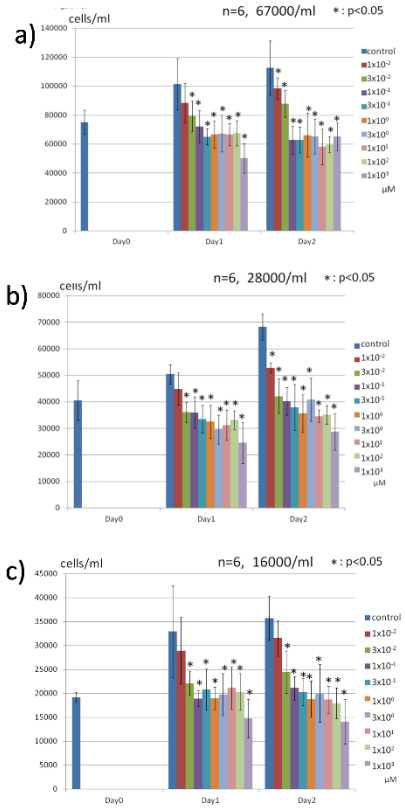


Figure 2

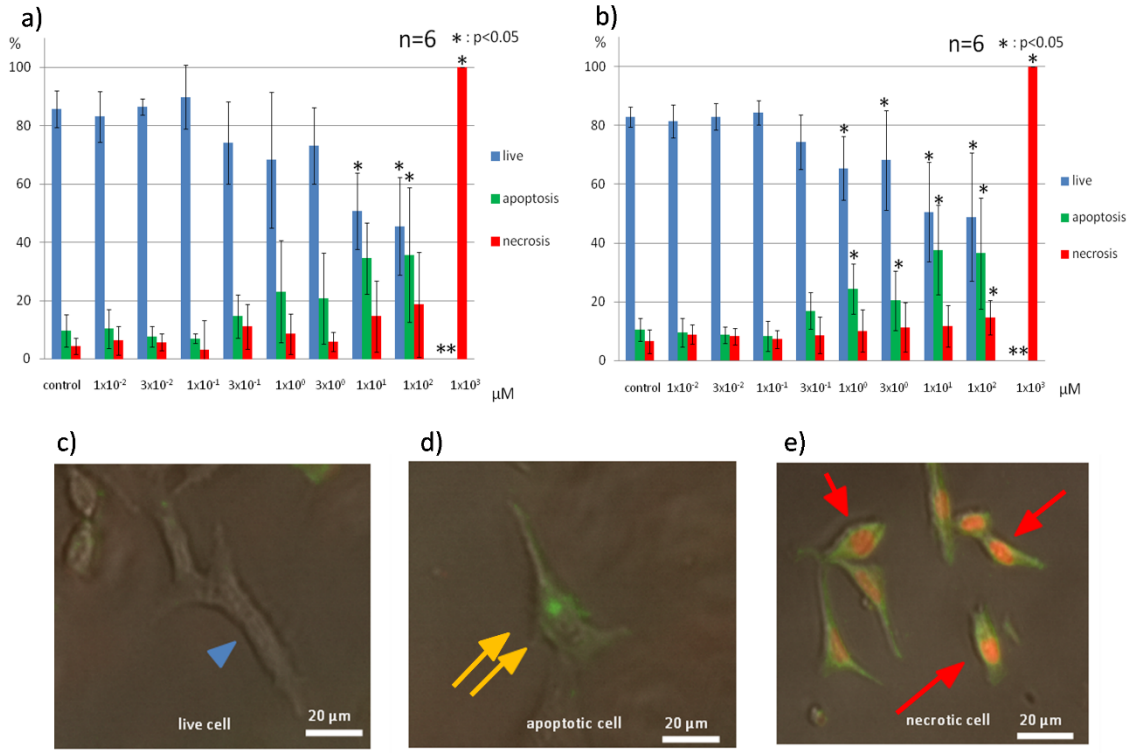


Figure 3

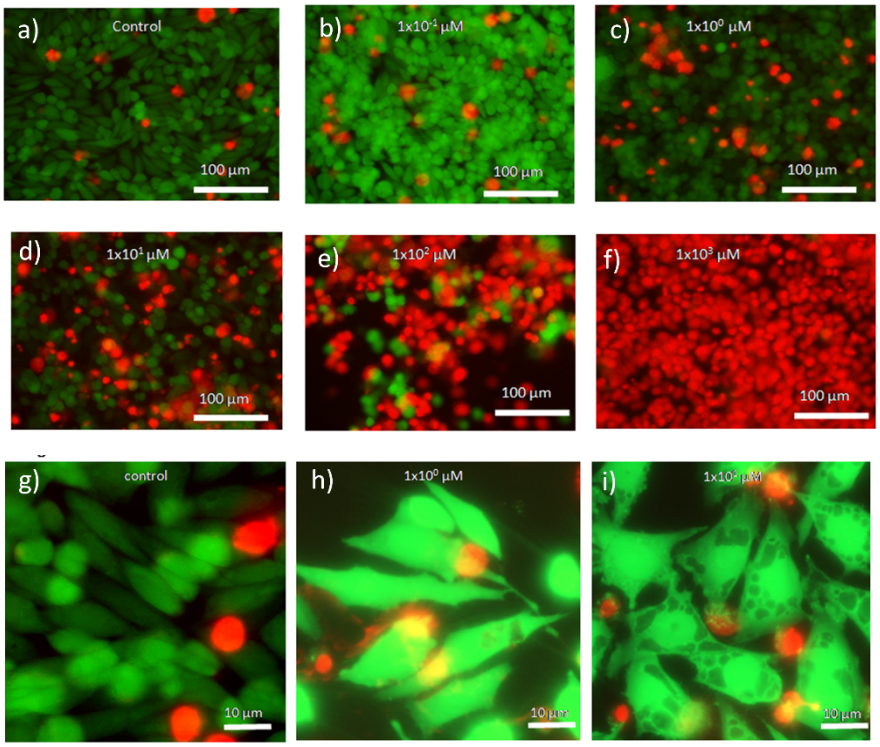
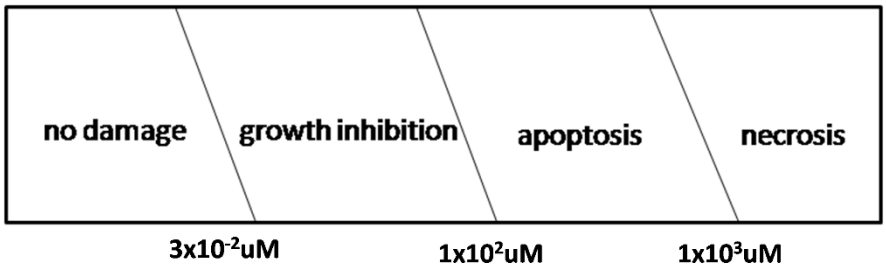


Figure 4

a) One day



b) Two days

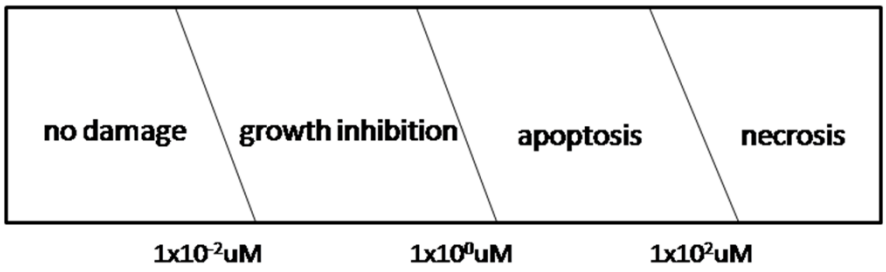


Figure 5

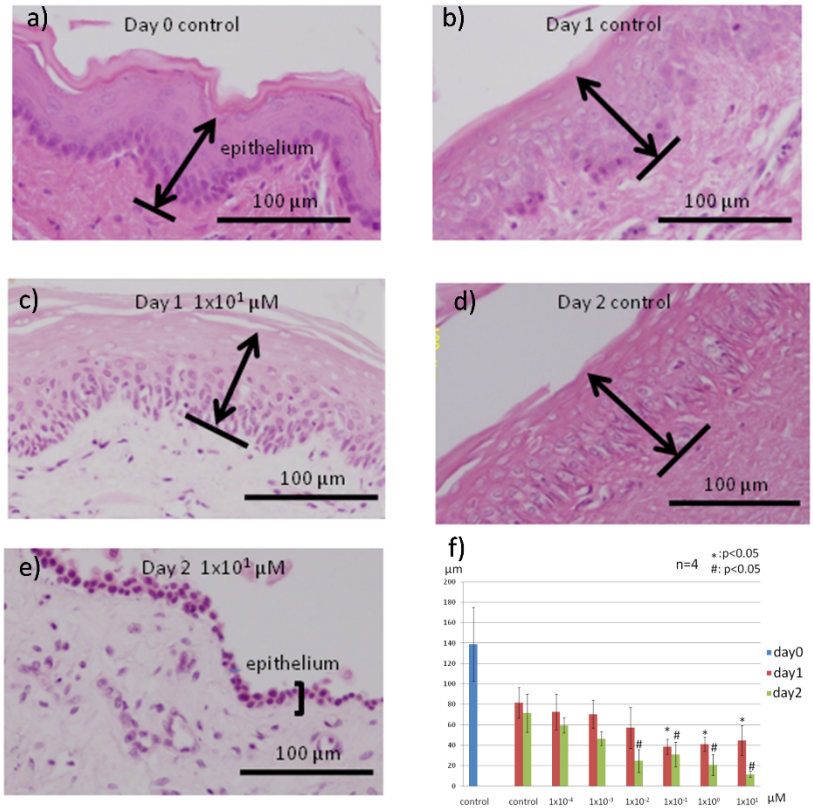


Figure 6